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METABOLIC ADAPTATION OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*, DURING EXERCISE AND ENVIRONMENTAL HYPOXIA AND SUBSEQUENT RECOVERY*

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Abstract

Metabolic responses to exercise, to exposure to environmental anaerobiosis, and to subsequent recovery were investigated in muscle, hepatopancreas, and hemolymph of the horseshoe crab, *Limulus polyphemus*. Exercise caused a considerable decline in arginine phosphate and the formation of D-lactate in muscle tissue, whereas the adenylate energy charge was maintained. Some of the D-lactate appears to have been transported from muscle tissue into the hemolymph. This occurred, apparently, during exercise as well as during recovery. Hemolymph postbranchial P_{O_2}, which decreased during exercise, and tissue phosphagen stores were rapidly restored to aerobic control values upon recovery, while D-lactate oxidation was protracted, especially in muscle.

Environmental anaerobiosis for 48 h was fueled by the breakdown of arginine phosphate (considerable only in muscle tissue) and glycogen, resulting in the accumulation of arginine, D-lactate, and alanine in both muscle and hepatopancreas. Alanine production may occur *via* glutamate-pyruvate transaminase and glutamate dehydrogenase, which take over the role of D-lactate dehydrogenase to maintain redox balance during the later phases of anaerobiosis. Recovery from anaerobiosis was characterized by a rapid replenishment of the phosphagen, a rapid drop in alanine concentration, and a protracted time-course for the decline in D-lactate levels, which was somewhat faster in the hepatopancreas than in muscle tissue.

Introduction

Hypoxia and even anoxia are encountered by many marine species. The horseshoe crab *Limulus polyphemus*, has successfully occupied microhabitats such as the estuarine intertidal zone and shallow tidal marshes, where low oxygen partial pressures may occur daily as well as a seasonally. This species is capable of extensive locomotion with its walking legs; smaller animals often swim by rhythmically moving their legs and gill leaflets (see Shuster, 1982). During excessive locomotory activity, oxygen consumption by these muscles probably exceeds the rate at which oxygen can be delivered by the circulatory system. Thus, under these conditions energy provisions are met by anaerobic metabolism (see Gäde, 1983).

Metabolic adaptations to both environmental and functional hypoxia have been studied to some extent in invertebrates, mostly molluscs, annelids, and crustaceans (for reviews see Schöttler, 1980; Gäde, 1983). During functional hypoxia, energy is typically provided by the breakdown of phosphagens (mostly arginine phosphate) and

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by anaerobic glycolysis, resulting in the production of octopine (or other opine compounds) or lactate (for reviews see Gäde, 1980; Gäde and Grieshaber, 1986). During environmental hypoxia, the phosphagen arginine phosphate again is important for ATP production. Additionally, Crustacea ferment glycogen and accumulate the classical end product L-lactate. However, in most annelids and molluscs, there is a simultaneous fermentation of glycogen and aspartate resulting in the accumulation of succinate and alanine during the first hours of hypoxia (de Zwaan and Dando, 1984). Later, when the aspartate pool is depleted, succinate production is derived exclusively from glycogen. This may be achieved by regulation at the phosphoenolpyruvate branchpoint (de Zwaan, 1977). The bulk of carbon flow is through phosphoenolpyruvate carboxykinase (PEPCK) rather than pyruvate kinase (PK). During prolonged periods of hypoxia, succinate is further metabolized to propionate.

Only a few investigations have examined the metabolic events immediately following environmental or functional anaerobiosis (see Ellington, 1983). These events include the recharging of the high energy phosphates, ATP and arginine phosphate, oxidation of end products, and resynthesis of anaerobic substrates.

Surprisingly, only scarce and scattered data on the above processes are available for the horseshoe crab. Arginine kinase is present in muscle (Blethen, 1972), indicating that arginine phosphate might contribute to energy production. The horseshoe crab possesses a D-lactate dehydrogenase (Long and Kaplan, 1968) which is present in a system of kinetically distinct, tissue-specific isoenzymes (Carlsson and Gäde, 1985). PK and PEPCK have been investigated in the tissues of *L. polyphemus* (Falkowski, 1974; Zammit and Newsholme 1978; Zammit *et al.,* 1978). The kinetic data suggest that the bulk of carbon flow is through PK rather than PEPCK (Zammit and Newsholme, 1978). Fields (1982) found that the bulk of label from anaerobic ^{14}C-glucose degradation by *in vitro* heart preparations was accumulated in lactate. Only trace amounts of radioactivity appeared in succinate.

Therefore in the present study we wanted to evaluate the overall capacity for anaerobic metabolism in muscle and hepatopancreas of *Limulus polyphemus* by estimating the relative maximal activities of enzymes of the intermediary metabolism. We were also interested in learning which metabolic events take place during exercise in muscle and hemolymph, and which take place during various periods of environmental hypoxia in muscle, hepatopancreas, and hemolymph. Finally, following the exercise stress and environmental hypoxia, we analyzed the metabolic changes in the three tissue compartments after recovery under aerobic conditions.

Materials and Methods

Animals and tissues

Specimens of the adult horseshoe crab, *Limulus polyphemus,* (20–25 cm prosoma width) were obtained live from the Marine Biological Laboratory (Woods Hole, MA), shipped by air freight to Bonn (FRG), and kept at 15°C ± 2°C, in artificial seawater (33‰ salinity), in aquaria of 300 l. Until four days before experimentation, the animals were fed frozen fish or mussel meat once a week.

Muscle tissue from two different sites were used in this study. First, for the exercise experiments, we analyzed the muscle that moves the gill leaflets. Second, due to its small mass and its possible contribution to contractions even under hypoxic conditions, the telson levator muscle was chosen for the experiments dealing with environmental anoxia and recovery. Furthermore, the activities of key enzymes were also measured in the levator muscle, but preliminary experiments indicated that they were very similar in the gill-muscle.

In addition, a portion of the hepatopancreas from the dorsal anterior part of the animal was analyzed for enzymatic activities and changes of metabolites during environmental anoxia and recovery.

Materials

Biochemicals were purchased from Sigma Chemical Company (Taufkirchen, FRG) and Boehringer GmbH (Mannheim, FRG). All other chemicals were of reagent grade quality and came from Merck (Darmstadt, FRG). D-lactate dehydrogenase from muscle tissue of *Limulus polyphemus* was purified as described elsewhere (Carlsson and Gäde, 1985) and was used to assay D-lactate concentrations in tissues and hemolymph.

Experimental procedure

Metabolic responses to work and recovery. Animals were removed from the aquaria for different experiments, at zero time, and subjected to various periods of exercise. Horseshoe crabs held vertically in water by their telsons attempt to right themselves by making swimming movements using their legs and gills; under the conditions chosen, about 45 to 55 gill movements were made per min. After a given period of exercise (24, 47, 96, 132, 161, and 240 s), animals were removed from the tank, and the muscles that move the gill leaflets were excised and frozen in liquid nitrogen. This procedure took about 20 s. After 4 min of exercise, some animals were left to recover in well-aerated seawater for various intervals, and treated as above. Also, a zero time group was selected that had done no work at all.

Other animals were exercised for 2, 4, and 10 min, and the D-lactate concentration in the hemolymph (postbranchial, taken through the arthrodial membrane between the prosoma and the opisthosoma) was measured before and after exercise as well as at various time intervals during recovery. In some of these animals, the P_{O_2} of the postbranchial hemolymph was determined with an oxygen electrode (E 5046, Radiometer; Copenhagen, Denmark) housed in a thermostated (12 ± 2°C) cell (D 616, Radiometer). The electrode was calibrated with air-saturated seawater and with water completely depleted of oxygen by the addition of Na_2SO_3 to give a 7% solution.

All tissues were stored at −25°C.

Metabolic responses to environmental hypoxia, exposure to air, and recovery. Horseshoe crabs were incubated in Plexiglas respiration chambers filled with about 15 l seawater at 12°C which had been gassed with pure nitrogen until P_{O_2} (monitored with an oxygen electrode) reached almost zero mm Hg. After the animals were inserted, the chamber was flushed with nitrogen for another 15 to 30 min before it was closed. The chamber was placed in a constant temperature room (12 ± 1°C) for the remainder of the experiment. At the end of different incubation periods, animals were removed, a postbranchial hemolymph sample was taken, and portions of the telson levator muscle and hepatopancreas were dissected, blotted on filter paper, and immediately frozen in liquid nitrogen. An aliquot of the hemolymph sample was immediately used for P_{O_2} determination (see above), and the rest was blown into perchloric acid (see below).

Another group of horseshoe crabs was incubated in the Plexiglas respiration chamber without any seawater at 12°C (air exposure). A third group of animals was exposed to air with their gill leaflets mechanically prevented from ventilating. This was achieved by binding the gill leaflets firmly onto the body with the help of metal strips fastened with rubber bands.

Biochemical analyses of tissue and hemolymph samples

Tissue samples were fragmented with a mortar and pestle chilled in liquid nitrogen. For each analysis, approximately 200 mg of tissue of an individual muscle was weighed and homogenized in 10 volumes (w:v) 6% perchloric acid (0–4°C). The homogenates were centrifuged at 19,000 × g for 20 min and the supernatant fluids neutralized with 5 M $KHCO_3$. The neutralized extract was centrifuged as above and the supernatant immediately used for the determination of the adenylates, arginine, and arginine-phosphate. Other metabolites were determined after storage of the extracts at −25°C.

ATP was determined according to Lamprecht and Trautschold (1974), ADP and AMP after Jaworeck *et al.* (1974), arginine by the method of Gäde and Grieshaber (1975), arginine phosphate according to Grieshaber and Gäde (1976), alanine after D. H. Williamson (1974), succinate after J. R. Williamson (1974), and D-lactate according to Gawehn and Bergmeyer (1974) using the D-lactate dehydrogenase from *Limulus polyphemus.* Using conspecific D-LDH is advantageous because of its much higher affinity for D-lactate than the commercially available enzyme preparation from *Lactobacillus* (Carlsson and Gäde, 1985). No interference with heavy metal ions was observed in our lactate assay as outlined previously (Gäde, 1984).

Hemolymph samples (100–300 mg) were blown into pre-weighed vials containing 0.5 ml 6% perchloric acid; the vials were weighed again, centrifuged as above, and the supernatants were neutralized with 5 M K_2CO_3. After centrifugation, the supernatant was used for the D-lactate determination.

All metabolite data were analyzed for significant changes by Student's t-test using confidence limits of $P \leqq 0.05$.

Profile of muscle and hepatopancreas enzyme activities

Activities of key enzymes of the intermediary metabolism were estimated in crude, desalted, cell-free extracts of the telson levator muscle and hepatopancreas of *L. polyphemus.* Approximately 1 g of tissue was cut into small pieces, resuspended in a 5-fold volume (w:v) of extraction buffer [100 mM triethanolamine-HCl (TRA) buffer containing 1 mM 2-mercaptoethanol at pH 7.6], homogenized by sonification (Branson sonifier), and centrifuged at 20,000 × g for 20 min. The supernatant was passed through a Sephadex G-25 column (Deutsche Pharmacia GmbH, Freiburg, FRG) to remove low molecular weight compounds.

The enzymes were assayed by standard procedures reported in the literature. The assay conditions have been outlined in detail previously (Meinardus-Hager and Gäde, 1986). Exceptions are the reaction mixtures (final concentration in a 1 ml cuvette) of the following enzymes:

Alcohol dehydrogenase (EC 1.1.1.1): 85.5 mM sodium pyrophosphate buffer pH 9, 19 mM glycine, 6.2 mM semicarbazid-hydrochloride, 0.6 M ethanol, 1.8 mM NAD^+, 1 mM glutathion.

D-lactate dehydrogenase (LDH, EC 1.1.1.28): 100 mM potassium phosphate buffer pH 7.0, 0.15 mM NADH, start with 2.5 mM pyruvate (1 mM pyruvate in case of extracts from hepatopancreas).

Glutamine synthetase (EC 6.3.1.2): 200 mM Tris/HCl pH 7.7, 100 mM hydroxylammoniumchloride, 10 mM Na_2HAsO_4, 0.5 mM $MnSO_4$, 1.5 mM ADP, 100 mM glutamine. After 30 min the reaction was stopped by addition of an acidic solution of $FeCl_3 \times 6\ H_2O$ and the extinction measured at 546 nm.

All assays were conducted in a filter photometer (Vitatron DCP 4) or a LKB Ultrospec recording spectrophotometer at 25°C. Assays were initiated by the addition of substrate.

Results

Profile of the activities of key enzymes in the intermediary metabolism

The activities of some enzymes involved in the energy metabolism in muscle and hepatopancreas tissue of *L. polyphemus* are listed in Table I. Glycogen phosphorylase in the muscle was only 6% in the a-form, while in the hepatopancreas the a-form of the enzyme represented 46% of the total enzyme activity. In the muscle tissue, the activity of the hexokinase was about 7-fold higher than that of the activated phosphorylase, whereas no significant difference between these two enzymes was observed in the hepatopancreas. Glyceraldehyde-3-phosphate dehydrogenase of the muscle had the highest activity of the enzymes of the Embden-Meyerhof pathway followed by pyruvate kinase and phosphofructokinase. This was true also for hepatopancreas, but

Table I

Activity of various enzymes in telson levator muscle and hepatopancreas from Limulus polyphemus

Enzyme	(n)	Enzyme activity (μmoles substrate conversion per min and g wet weight)	
		Telson levator muscle	Hepatopancreas
Enzymes involved in glycolysis:			
Phosphorylase a-form	(2)	0.022 ± 0.005	0.13 ± 0.03
a + b-form		0.34 ± 0.10	0.28 ± 0.06
Hexokinase	(4)	2.32 ± 0.45	0.39 ± 0.16
Phosphofructokinase	(3)	29.9 ± 6.2	1.22 ± 0.12
Glyceraldehydephosphate dehydrogenase	(3)	136 ± 12	10.5 ± 2.9
Pyruvate kinase	(3)	73.3 ± 13	5.6 ± 1.4
Lactate dehydrogenase	(4)	102 ± 1	29.3 ± 5.1
Octopine dehydrogenase	(3)	n.d.*	n.d.
Strombine dehydrogenase	(3)	n.d.	n.d.
Alanopine dehydrogenase	(3)	n.d.	n.d.
Alcohol dehydrogenase	(3)	n.d.	n.d.
Enzyme involved in phosphagen utilization:			
Arginine kinase	(3)	616 ± 17	34.0 ± 9.6
Citric acid cycle enzymes:			
Citrate synthase	(2)	2.05 ± 0.28	1.49 ± 0.08
Malate dehydrogenase	(4)	156 ± 15	91 ± 7
Enzymes involved in amino acid metabolism:			
Glutamate dehydrogenase			
NADH-dependent	(4)	1.74 ± 0.24	0.69 ± 0.12
NADPH-dependent	(4)	n.d.	n.d.
Glutamine synthetase	(2)	0.027 ± 0.008	0.081 ± 0.013
in the presence of 10 m*M* alanine	(2)	0.018 ± 0.005	0.005 ± 0.016
Glutamate-oxaloacetate transaminase	(3)	28.9 ± 3.7	19.1 ± 7.7
Glutamate-pyruvate transaminase	(3)	13.5 ± 1.0	16.7 ± 2.4
Enzymes involved in gluconeogenesis:			
Fructose diphosphatase	(3)	0.74 ± 0.19	2.04 ± 0.18
Phosphoenolpyruvate carboxykinase	(3)	1.5 ± 0.3	2.1 ± 0.5

* n.d. = not detectable.

the maximum activities were 10- to 20-fold lower than in muscle tissue. Of the four terminal pyruvate reductases measured in both tissues, only D-lactate dehydrogenase was present. There was no activity at all of octopine-, alanopine-, and strombine dehydrogenase. The activity of D-lactate dehydrogenase in muscle tissue was almost as high as the activity of glyceraldehyde-3-phosphate dehydrogenase and, in hepatopancreas, 3-fold of the latter activity.

Fermentation of glycogen to ethanol occurs in anoxic muscle of fishes (Shoubridge and Hochachka, 1980) and in tissues of larvae of the insect *Chironomus thumii thumii* (Wilps and Zebe, 1976). Due to the absence of activity of alcohol dehydrogenase in tissues of *L. polyphemus,* this type of fermentation cannot take place.

Arginine kinase activity was very high in muscle tissue, but the hepatopancreas displayed only a fraction of this activity. Malate dehydrogenase activity was very high in both tissues, and there was a significant activity of citrate synthase in both tissues.

Activity of phosphoenolpyruvate carboxykinase, which catalyses the fixation of CO_2, could readily be measured in both tissues, but activity was slightly higher in the hepatopancreas. This was also true for the enzyme fructose-biphosphatase, an enzyme thought to operate during gluconeogenesis.

Enzymes involved in amino acid metabolism were also assayed in both tissues. The transaminases showed the highest activities. Glutamate dehydrogenase in both tissues was only active with NADH, and not NADPH, as the co-substrate. Very low activities of glutamine synthetase were found in both tissues (but they were higher in hepatopancreas); this enzyme was inhibited by the addition of 10 m*M* alanine to the reaction mixture.

Metabolic responses to exercise and recovery

When specimens of *L. polyphemus* were subjected to exercise, there was a linear relationship between the numbers of gill movements and time (Fig. 1, inset). The level of D-lactate in the gill-muscle tissue increased linearly until up to 3 min and then plateaued (Fig. 1). After 20 and 30 min of recovery from 4 min of exercise, the D-lactate levels were significantly lower than the exercise value, but the control levels were not attained. There was no change in the alanine concentration during work (Fig. 1). Exercise also had no significant effect on the adenylates in the muscle tissue and, consequently, the adenylate energy charge did not change (Table II). In contrast, arginine phosphate levels fell significantly during exercise (Fig. 2). The most pronounced change occurred during the first 47 s of exercise. The increase in free arginine levels was a mirror image of the pattern of change in arginine phosphate levels (Fig. 2). During recovery, arginine phosphate levels rose rapidly and reached initial levels after about 20 to 30 min. At the same time there was a decrease in the arginine concentration. There were no changes in aspartate and succinate levels in any of these experimental treatments (results not shown).

In the hemolymph, D-lactate concentrations did not change during 2 min of exercise, whereas concentrations rose significantly during recovery (Fig. 3): the highest D-lactate concentrations were observed after 5 min of recovery. After 90 min of recovery, hemolymph D-lactate concentrations were not different from controls. After 4 and 10 min of exercise, the D-lactate concentrations in the hemolymph were significantly increased above control concentrations, however, the concentrations at the two times were not significantly different from each other. During recovery from 4 and 10 min of work, there was an apparent large increase of the D-lactate concentration in the hemolymph; however, due to the inherent variability of the measurement, this increase was not significantly different from the values obtained just after work.

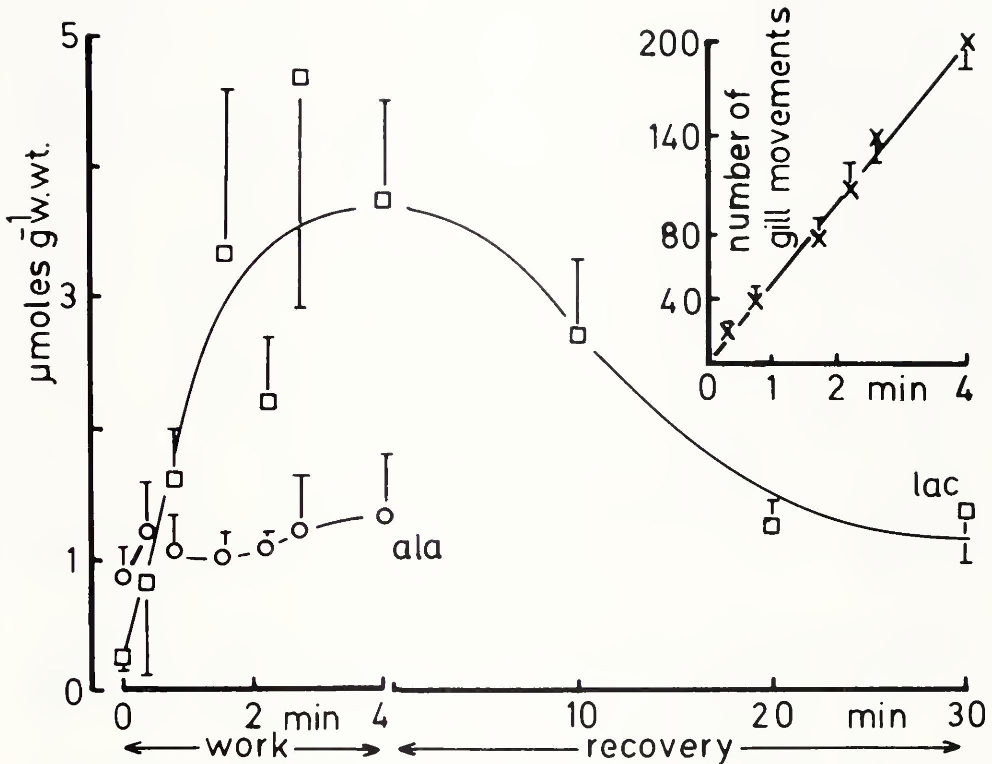

FIGURE 1. Alterations in the levels of D-lactate (□ — □) and alanine (O — O) in muscle that moves the gill leaflets of *Limulus polyphemus* during exercise and recovery. Each value is a mean ± SD (n = 4). The inset shows the relationship of the numbers of gill movements *versus* time.

P_{O_2} levels of the hemolymph decreased after 4 min of exercise, but were already back to control levels after 10 min of recovery (Fig. 4).

Metabolic responses to environmental hypoxia, exposure to air, and recovery

In a first series of experiments we analyzed the oxygen partial pressure of postbranchial hemolymph and the blood D-lactate concentration after subjecting specimens

TABLE II

Alterations in the levels of adenylates (µmoles per g wet weight) and the calculated energy charge in muscle that moves gill leaflets of Limulus polyphemus *during different durations of exercise*

Time (s)	(n)	ATP	ADP	AMP	Sum	Energy charge
0	3	2.73 ± 0.60	0.42 ± 0.12	0.03 ± 0.01	3.18	0.92
23.6 ± 4.5	3	2.47 ± 0.49	0.42 ± 0.16	0.06 ± 0.03	2.95	0.91
46.7 ± 7.9	3	3.24 ± 0.72	0.51 ± 0.15	0.04 ± 0.01	3.95	0.92
95.6 ± 9.0	3	2.60 ± 0.53	0.44 ± 0.10	0.03 ± 0.02	3.07	0.92
161.0 ± 8.2	3	2.56 ± 0.24	0.48 ± 0.14	0.04 ± 0.01	3.08	0.91
240.5 ± 26.7	3	2.44 ± 0.44	0.46 ± 0.19	0.05 ± 0.01	2.95	0.91

Each value is a mean ± SD.

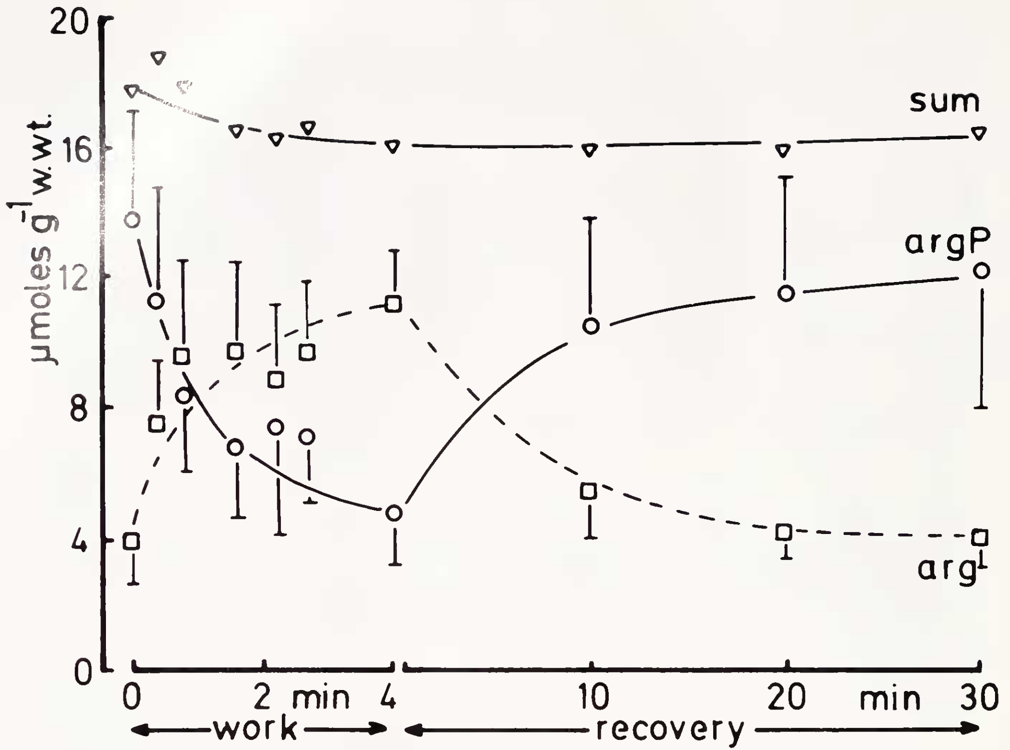

FIGURE 2. Changes in the levels of arginine phosphate (O — O) and arginine (□ — □) and the sum of these metabolites (∇ — ∇) in muscle that move the gill leaflets of *Limulus polyphemus* during exercise and recovery. Each value is a mean ± SD (n = 5).

of *L. polyphemus* to extremely hypoxic seawater for various periods of time (Fig. 5A); and during air exposure for 72 h (Fig. 5B); and air exposure for 48 h when the gills were mechanically prevented from ventilating (Fig. 5C).

Incubation in oxygen-free seawater and exposure to air with unventilated gills produced the same general results (Fig. 5A *versus* 5C): postbranchial blood P_{O_2} decreased to low levels after only a short incubation period, and blood D-lactate levels rose to about 3 µmoles/g wet weight of hemolymph during the first two hours and stayed almost contant thereafter. During air exposure with intact gills (Fig. 5B), a decrease in postbranchial P_{O_2} was observed after 2 h, but thereafter it rose again to about 30 mm Hg. D-lactate levels increased somewhat during the first 2 h but were back to control levels after 48 h of air exposure. During recovery from a 48 h period of incubation in oxygen-free seawater (Fig. 5A), P_{O_2} levels reached control values within 30 min of recovery, and after 1 and 2 h of recovery were slightly higher than P_{O_2} values observed under control conditions. The hemolymph D-lactate levels showed a trend towards an increase during the first hour of recovery (Fig. 5A), but due to the high variation this was not significant. After 8 h of recovery, the D-lactate levels were still higher than control values.

Incubation in oxygen-free seawater had no significant effect on the adenylate concentrations and the energy change in muscle, but ATP levels fell and ADP levels rose significantly in hepatopancreas after 8 h of incubation (Table III). After 48 h of hypoxia

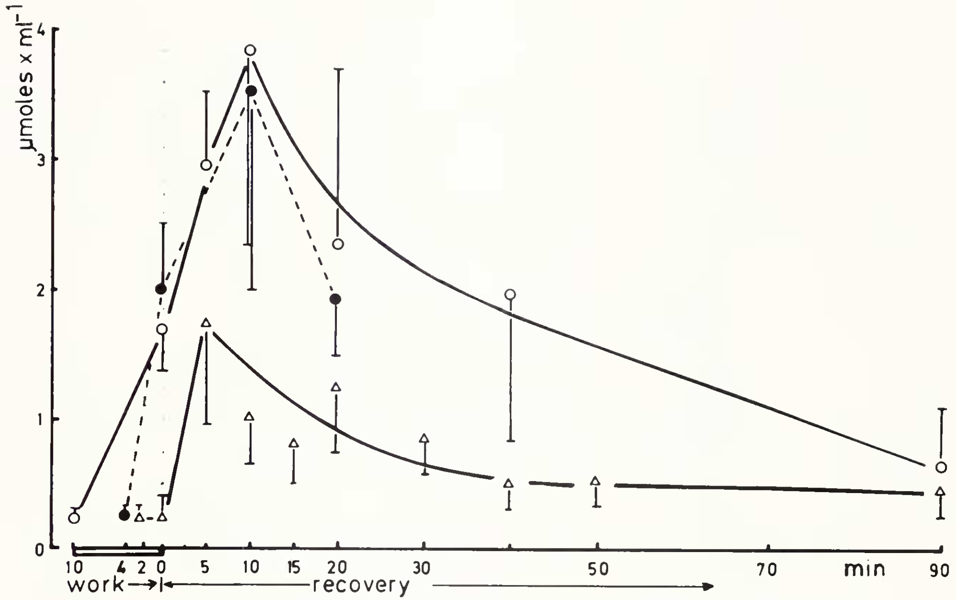

FIGURE 3. Time course of the levels of D-lactate in the hemolymph of *Limulus polyphemus* during different durations of exercise (2 min: △ — △; 4 min: ● — ●; 10 min: ○ — ○) and subsequent recovery. Each value is a mean ± SD (n = 4).

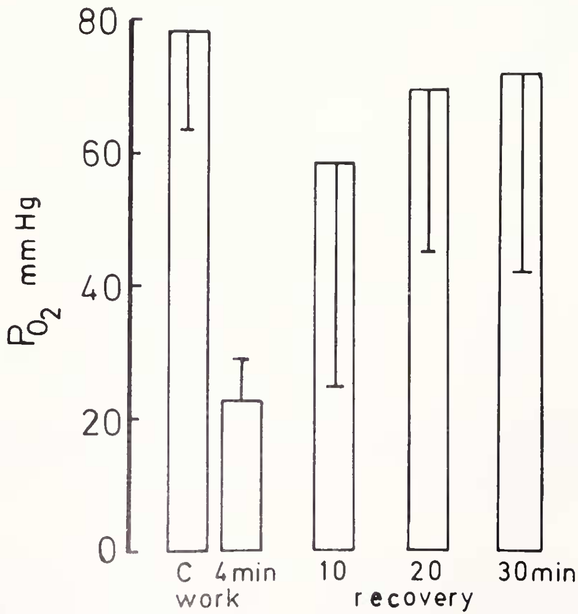

FIGURE 4. Changes in postbranchial P_{O_2} in the hemolymph of *Limulus polyphemus* during work and recovery. Each value is a mean ± SD (n = 4).

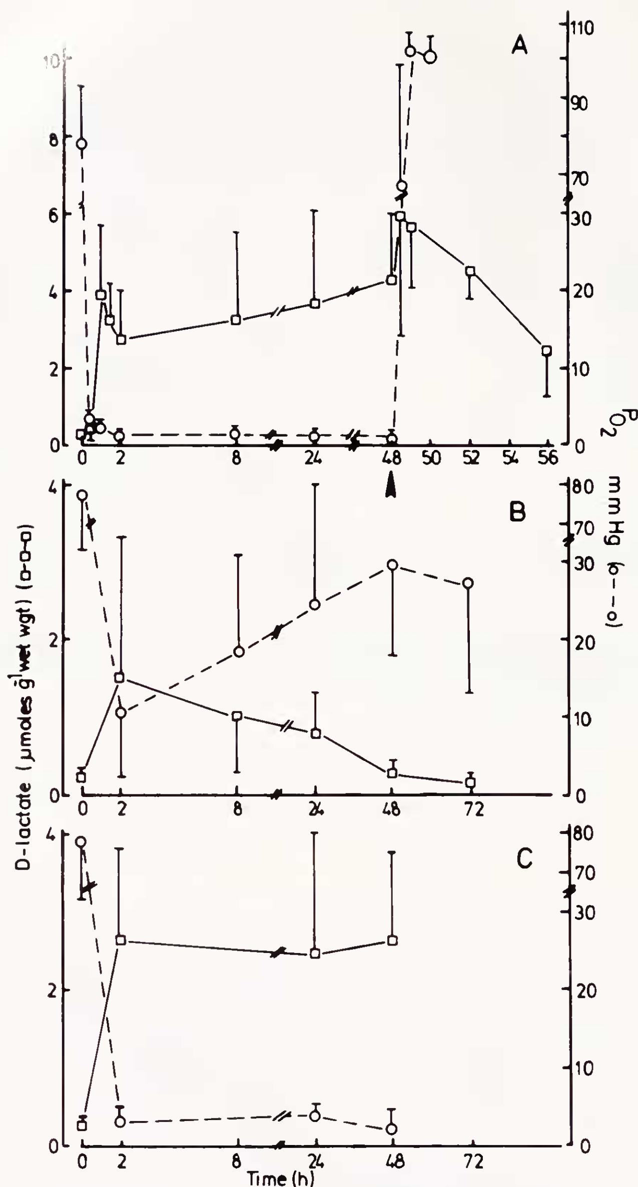

FIGURE 5. Effect of incubation in oxygen-free seawater (A), air exposure (B) and air exposure with mechanically closed gills (C) as well as recovery (A only) therefrom of the level of hemolymph D-lactate (□ — □) and postbranchial P_{O_2} (○ — ○) in *Limulus polyphemus*. Onset of recovery is indicated by an arrow. Each value is a mean ± SD (n = 4).

only one third of the control ATP levels remained, while ADP levels were 3-fold above the control levels. The adenylate energy charge fell from 0.86 (control) to 0.61 after 48 h of hypoxia. In the muscle, arginine phosphate levels fell significantly during hypoxia with the greatest changes occurring in the first 10 h (Fig. 6). Although there

TABLE III

Effect of incubating Limulus polyphemus *in oxygen-free seawater on the levels of adenylates (μmoles per g wet weight) and the calculated energy charge in the telson levator muscle and hepatopancreas*

Time (h) of anoxia	Telson levator muscle					Hepatopancreas				
	ATP	ADP	AMP	Sum	Energy charge	ATP	ADP	AMP	Sum	Energy charge
0.0	2.82 ± 0.65	0.48 ± 0.14	0.05 ± 0.21	3.35	0.91	0.47 ± 0.12	0.10 ± 0.02	0.03 ± 0.01	0.60	0.86
0.5	2.69 ± 0.26	0.59 ± 0.21	0.05 ± 0.02	3.33	0.90	0.33 ± 0.05	0.09 ± 0.05	0.01 ± 0.01	0.44	0.86
2.0	2.43 ± 0.20	0.53 ± 0.16	0.06 ± 0.03	3.02	0.89	0.37 ± 0.11	0.13 ± 0.02	0.03 ± 0.01	0.53	0.82
4.0	2.98 ± 0.43	0.60 ± 0.07	0.06 ± 0.01	3.64	0.90	0.35 ± 0.14	0.11 ± 0.03	0.04 ± 0.01	0.50	0.81
8.0	2.80 ± 0.13	0.67 ± 0.06*	0.04 ± 0.02	3.51	0.89	0.27 ± 0.08*	0.24 ± 0.04*	0.02 ± 0.01	0.42	0.74
24.0	2.73 ± 0.49	0.79 ± 0.04*	0.05 ± 0.02	3.57	0.88	0.26 ± 0.04*	0.24 ± 0.09*	0.03 ± 0.01	0.53	0.72
48.0	2.81 ± 0.18	0.67 ± 0.07*	0.07 ± 0.02	3.55	0.89	0.14 ± 0.02*	0.28 ± 0.12*	0.04 ± 0.02	0.46	0.61

* Significantly different to control value.
Each value is a mean ± SD (n = 4).

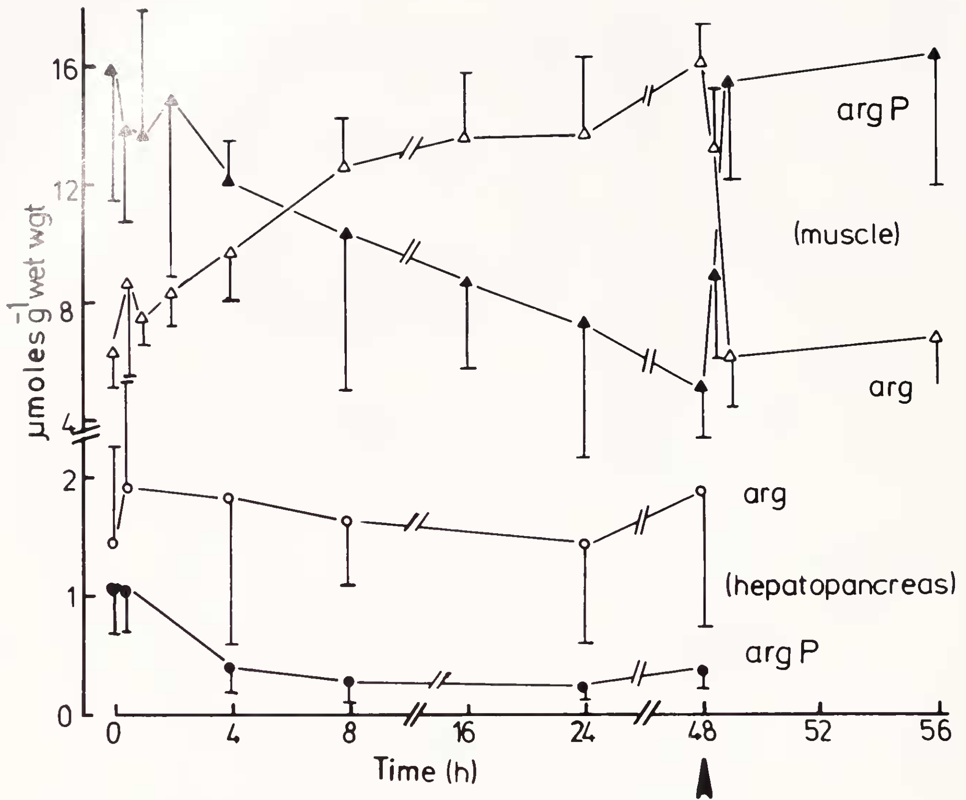

FIGURE 6. Effect of incubating *Limulus polyphemus* in oxygen-free seawater for various times on the levels of arginine phosphate and arginine in the telson levator muscle and hepatopancreas. The time course of changes occurring during recovery in muscle is also shown; onset of recovery is indicated by the arrow. Each value is a mean ± SD (n = 5).

was some breakdown of arginine phosphate in the hepatopancreas during hypoxia, quantitatively this did not contribute much to overall energy production due to the low initial levels of this compound (Fig. 6). During recovery after hypoxia, arginine phosphate levels in muscle returned to initial values within 1 h.

There were significant changes in the levels of D-lactate and alanine during hypoxia in both muscle and hepatopancreas tissue. After an initial increase in the first hour, D-lactate levels in muscle tissue as well as in hepatopancreas seemed to increase slightly with time, but due to the high variability these changes were not significant (Fig. 7). Until 2 h of recovery had passed, D-lactate levels in muscle remained high, and they were still above control levels after 8 h of recovery. In the hepatopancreas, the decrease in D-lactate levels was somewhat faster, yet control values were not reached after 8 h of recovery.

L-alanine levels increased with time of hypoxia in both tissues (Fig. 8). However, in muscle tissue most of the alanine was produced until 24 h of hypoxia, whereas there was a further increase in alanine levels between 24 and 48 h of hypoxia in the hepatopancreas. Succinate production was minimal during the first 10 h of hypoxia in the muscle tissue and was absent in hepatopancreas (Fig. 8). Aspartate levels of

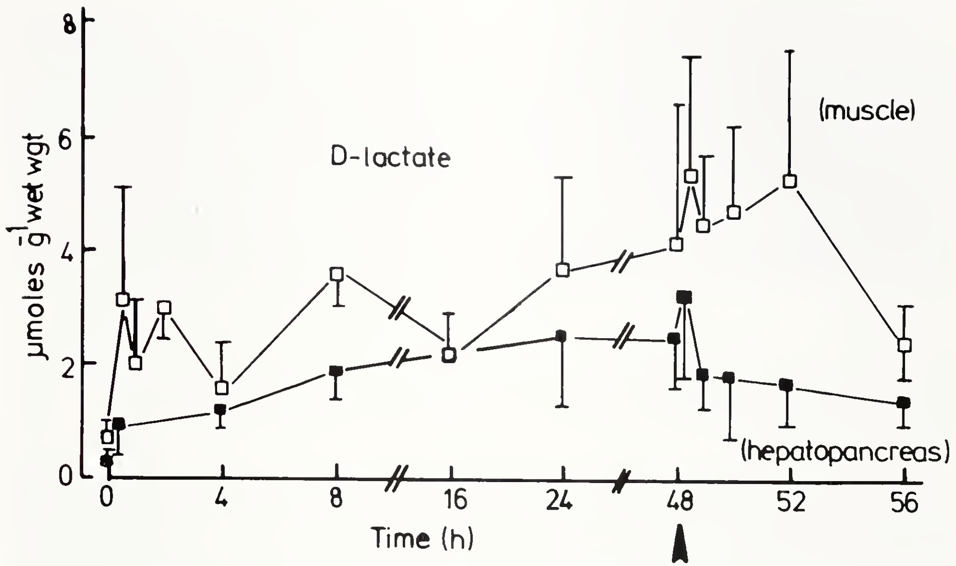

FIGURE 7. Effect of incubating *Limulus polyphemus* in oxygen-free seawater for various times on the level of D-lactate in the telson levator muscle and hepatopancreas. The time course of changes occurring during recovery is also shown; onset of recovery is indicated by the arrow. Each value is a mean ± SD (n = 4).

muscle and hepatopancreas were 0.29 ± 0.11 and 0.24 ± 0.06 μmoles/g wet weight, respectively, in control animals and 0.13 ± 0.06 and 0.07 ± 0.04 μmoles/g wet weight, respectively, after 48 h of hypoxia (for all data: n = 4).

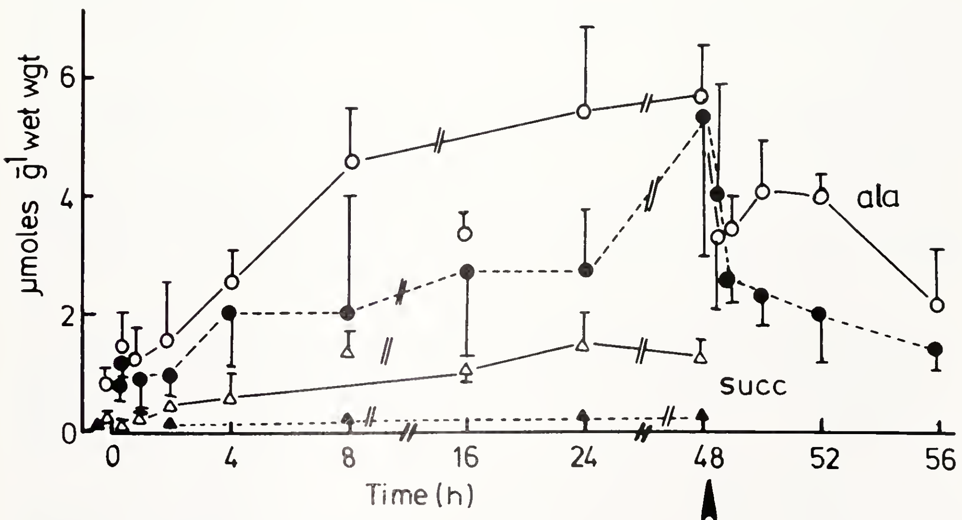

FIGURE 8. Effect of incubating *Limulus polyphemus* in oxygen-free seawater for various times on the level of alanine (○, ●) and succinate (△, ▲) in the telson levator muscle (open symbols) and hepatopancreas (closed symbols). The time course of changes in alanine levels occurring during recovery is also shown; onset of recovery is indicated by the arrow. Each value is a mean ± SD (n = 4).

During recovery from hypoxia, alanine levels fell in both muscle and hepatopancreas. However, the time course of alanine removal was much more rapid in the hepatopancreas (Fig. 8). The alanine concentration in the postbranchial blood was below 0.05 μmoles/g wet weight throughout the whole experiment (hypoxia and recovery). Muscle, hepatopancreas, and hemolymph glutamate levels (1.59 ± 0.58, 3.30 ± 1.22, 0.04 ± 0.02 μmoles/g wet weight, respectively) did not change significantly during the experiment, whereas muscle and hepatopancreas glutamine levels decreased significantly from 1.21 ± 0.42 and 2.84 ± 1.13 μmoles/g wet weight, respectively, to 0.33 ± 0.21 and 0.55 ± 0.03 μmoles/g wet weight, respectively. Control levels of glutamine in both tissues were not attained again until after 8 h of recovery.

Discussion

Scope of aerobic and anaerobic energy metabolism as estimated by maximal enzyme activities

The capacity for aerobic and anaerobic energy metabolism of the telson levator muscle and hepatopancreas of *L. polyphemus* was first estimated by the determination of relative enzyme activities of the intermediary metabolism. Such a comparison may be instructive in trying to understand potential differences in the metabolism of *L. polyphemus* muscle and hepatopancreas tissue. In muscle tissue, arginine phosphate and glucose primarily and glycogen to a lesser extent, may be considered fuels for energy production. This is indicated by the following data. The enzyme arginine kinase is present in very high catalytic activities. In fact, the activity is substantially higher than the glycolytic enzyme activities. Accordingly, the phosphagen can be broken down quite rapidly, even when the energy demand is much enhanced as it is during muscular activity. Although glycogen phosphorylase (and therefore glycogen breakdown) can be activated about 16-fold by AMP, hexokinase activity still is about 7-fold higher. This may indicate that during periods of enhanced energy demand, exogenous glucose from the hemolymph may be a more important carbon source than glycogen.

In the hepatopancreas the enzyme data suggest that glycogenolysis and glucose metabolism may contribute equally to energy production, whereas the breakdown of phosphagen plays only a minor role.

In both tissues, especially in muscle, the other glycolytic enzymes known to catalyse rate-limiting reactions, phosphofructokinase (PFK) and pyruvate kinase (PK) have high activities, and exceed that of phosphorylase from 4- to 31-fold. This indicates, that the initial enzymes of glycolysis (phosphorylase, hexokinase) most probably catalyze the reactions which are rate-limiting steps in this pathway.

The expected anaerobic end-product is D-lactate. Both tissues contain only this terminal dehydrogenase and the activity is quite high, even in the hepatopancreas.

A representative enzyme of the citric acid cycle, citrate synthase, is present in both tissues at activity levels which are much higher than observed in most anoxia-tolerant invertebrate tissues (see, for example, Pörtner *et al.,* 1984; Meinardus-Hager and Gäde, 1986) suggesting a high aerobic capacity.

The relatively high activities of enzymes thought to be involved in gluco- and glyconeogenesis, phosphoenolpyruvate carboxykinase, and fructose-bisphosphatase, indicate that this pathway might be working during recovery after anaerobiosis to recharge the depleted stores of glycogen and glucose as was indeed shown recently (Gäde *et al.,* 1986).

Energy metabolism during swimming and recovery

The significance of the above discussed enzymatic patterns for the anaerobic production of energy was shown in individual horseshoe crabs which were made to swim for different periods. During exercise, the aerobic capacity of the muscle tissue was not sufficient to meet the enhanced energy demand. Pronounced anaerobic synthesis of ATP was achieved by the production of D-lactate and, second, by the breakdown of the phosphagen. This rate of anaerobic ATP production appears to have been sufficient to meet the energy demands as shown by the unchanged energy charge. In crustaceans, however, the energy charge is lowered during vigorous activity (Onnen and Zebe, 1983; Gäde, 1984).

D-lactate produced in muscle tissue was only accumulated until a concentration of about 3–4 μmoles per g wet weight was reached, which occurred after only 2 min of work. During prolonged exercise muscle-produced D-lactate appears to have been released into the hemolymph. Unexpectantly, the hemolymph D-lactate concentration was not significantly different after 4 and 10 min work. This was also true during recovery after these periods of exercise, when in both cases a further increase of D-lactate was measured. The lack of difference between these exercised groups could be due to variability in the data resulting from the measurement of a transient in blood. Alternatively, the work output between 4 and 10 min may be lowered and, thus, aerobic energy production may contribute to a greater extent.

Oxidation of D-lactate during recovery is very protracted, so tissues other than muscle may be contributing. The hepatopancreas, for example, contains D-LDH isoenzymes, which are kinetically well suited for lactate oxidation (Carlsson and Gäde, 1985). In contrast, replenishment of pre-work arginine phosphate levels during recovery is rapid and probably achieved aerobically due to the effectiveness of the circulatory system.

Energy metabolism during environmental hypoxia, exposure to air and recovery

Incubation in oxygen-free seawater and air exposure with mechanically closed gills induce horseshoe crabs to rely on anaerobic metabolism for their total energy production; the hemolymph P_{O_2} declined to about zero and D-lactate levels increased. As no differences are found between these two anaerobic groups it is clear that excretion of D-lactate does not occur during incubation in oxygen-free water. Air exposure with intact gill function was tolerated for at least three days without significant D-lactate production, indicating the absence of anaerobic metabolism. The reason lies partly in the function of the gills and partly in the unique properties of the oxygen carrier, hemocyanin. Oxygenated hemocyanin-transport was calculated to be enhanced during air exposure by 24% due to the reverse Bohr-shift (Mangum *et al.,* 1975; Mangum, 1983).

In the telson levator muscle, the anaerobic energy metabolism was efficient enough to meet the energy demand during severe hypoxia for 48 h. There was no change in the levels of adenylates and, consequently, of the energy charge. ATP production was achieved by transphosphorylation of the phosphagen and breakdown of glycogen resulting in the production of D-lactate.

In contrast, due to the low initial concentrations of arginine phosphate, the energy charge decreased considerably in the hepatopancreas. Again, ATP was provided by the formation of D-lactate. However, in both tissues, the amount of D-lactate which accumulated never exceeded 4 μmoles per g wet weight, suggesting that another end product was formed by another route of anaerobic energy production.

A likely candidate was the succinate/propionate route well-known from euryoxic bivalves and annelids (for reviews see Gäde, 1983; Schöttler, 1980), but not operating to a meaningful extent in crustaceans. However, the succinate levels in muscle and hepatopancreas tissue were only elevated by 1 and 0.1 μmol/g wet weight, respectively, and propionate was not present at all. Other data support the notion that the succinate/propionate pathway is not working in *L. polyphemus* (*e.g.*, Fields, 1982; Zammit and Newsholme, 1978).

Which other route of energy production, besides arginine phosphate transphosphorylation and D-lactate formation, might occur then in *L. polyphemus?* Muscle and hepatopancreas accumulate L-alanine consistently. The bulk of this accumulation is observed after the main D-lactate formation has already occurred, and it quantitatively exceeds the D-lactate accumulation. Alanine production, *via* GOT and GPT, is known in bivalves and annelids, but is not possible in *L. polyphemus* due to the low levels of aspartate. We therefore propose a mechanism with the participation of GPT and glutamate dehydrogenase (see Fig. 9). Pyruvate derived by anaerobic breakdown of glycogen would be converted to alanine and α-ketoglutarate by the action of GPT. The keto acid, in turn, is oxidized with NH_4^+ by glutamate dehydrogenase to regenerate glutamate for the GPT reaction and to produce the oxidized coenzyme NAD^+. The

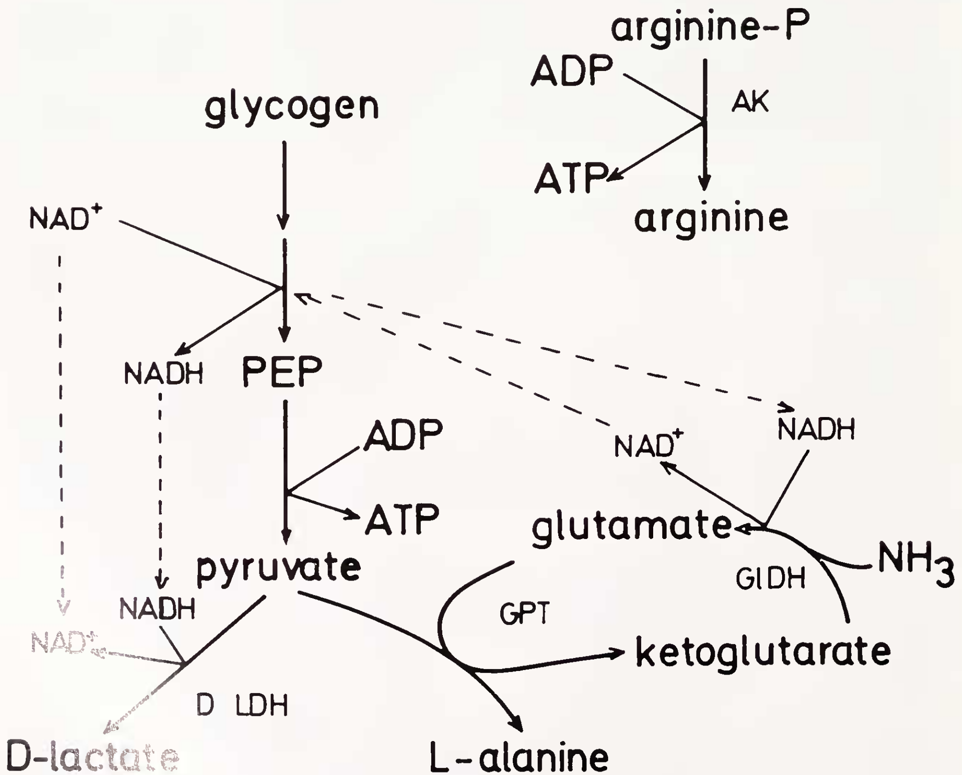

FIGURE 9. Proposed scheme of anaerobic energy metabolism in *Limulus polyphemus.* Note: no compartmentation (cytosol-mitochondria) is given.

only end product of this reaction would be alanine. Our experimental data support this hypothesis, as follows.

(1) The maximal activities of GPT and GluDH are sufficient to account for the observed rates of accumulation of alanine.

(2) Partially purified GluDH from *L. polyphemus* muscle tissue is exclusively NADH-, but not NADPH-dependent. In addition, GluDH and D-LDH from *L. polyphemus* appear to have similar catalytic affinities for NADH (Carlsson and Gäde, 1985, and unpub.).

For this reaction scheme to operate, GluDH must be localized in the cytoplasm where it is stoichiometrically coupled with the glyceraldehyde-3-phosphate dehydrogenase reaction. As yet, however, we have no data on the localization of GluDH in *L. polyphemus* tissue.

What is the advantage for the horseshoe crab to rely on alanine production via GluDH instead of a continuous formation of D-lactate? D-LDH is an equilibrium enzyme. The K_m-values for D-lactate are in the range of 10 m*M* for the muscle isoenzymes and even lower, 5 m*M*, for the hepatopancreas isoenzymes (Carlsson and Gäde, 1985). Thus, from a kinetic point of view, D-lactate cannot accumulate to high levels, since the reaction is shifted back then to D-lactate oxidation. In contrast, the K_m-value for L-lactate in the crayfish *O. limosus* is about 40 m*M* (Urban, 1969), and here we found L-lactate levels as high as 16 m*M* in muscle tissue after anaerobiosis (Gäde, 1984). In *L. polyphemus*, D-lactate production occurs mainly during the beginning of anaerobiosis; but later, when the D-lactate levels may hamper further reduction of pyruvate, NADH oxidation is achieved by the action of GluDH. The production of alanine may serve at least two purposes:

(1) The enzyme pyruvate kinase is inhibited by 85% in the presence of 5 mM alanine (Zammit and Newsholme, 1978; our unpub. results), and, therefore, glycolytic flux is reduced during prolonged anaerobiosis, as observed in bivalves and annelids (see, Gäde, 1983; Schöttler, 1980). Calculations of rates of anaerobic energy production in muscle tissue of *L. polyphemus* from our metabolic data (1.5 μmol of ATP per μmol of D-lactate and L-alanine produced) revealed a rate of 0.0465 μmol ATP/g wet weight/min during the first two hours of anaerobiosis, in contrast to 0.0036 during 16 to 48 hours of anaerobiosis.

(2) In mammals, the enzyme glutamine synthetase is inhibited by alanine (see Meister, 1974). This enzyme synthesizes glutamine from glutamate and NH_{4^+} coupled with the consumption of ATP. Glutamine, in turn, is important for the synthesis of, for example, purines, pyrimidines, carbamylphosphate, and various proteins. Inhibition of this enzyme, therefore, would channel glutamate and NH_{4^+} away and hamper the energy-expending synthesis of nitrogen compounds. Our preliminary data on crude extracts of horseshoe crab muscle and hepatopancreas glutamine synthetase showed inhibition by alanine and is thus in agreement with the arguments above. During recovery of *L. polyphemus* from 48 hours of anaerobiosis a rapid drop in the levels of alanine in muscle and hepatopancreas might relieve the inhibition of both glycolysis and glutamine synthetase.

In summary, the horseshoe crab is well equipped to cope with environmental anaerobiosis. Metabolic energy is derived by the breakdown of phosphagen and by the production of D-lactate and, later, alanine. In the latter phase GluDH may take over the role of D-LDH to maintain redox-balance. In contrast, aside from the transphosphorylation reaction, crustaceans use only anaerobic glycolysis leading to L-lactate accumulation as means of providing energy during low oxygen tension.

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